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ORIGINAL ARTICLE

Food Allergy And Gastrointestinal Disease

Bovine γ-globulin, lactoferrin, and lactoperoxidase are relevant bovine milk allergens in patients with α-Gal syndrome

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Abstract

Background: Mammalian meat is the most common trigger of the allergic reactions in patients with α-Gal syndrome (AGS). Milk and dairy, although less often, also cause a significant number of allergic manifestations. The aim of this study was to identify α-Gal-containing bovine milk proteins with allergenic properties among AGS patients.

Methods: Thirty-eight AGS patients with IgE to milk were included in the study. Milk proteins were analyzed for the presence of α-Gal and for binding by patients' IgE using immunoblot, ImmunoCAP, and inhibition ELISA. Allergenicity of milk and milk proteins was assessed by basophil activation test.

Results: More than half of the AGS patients reported allergic reactions to milk or dairy products. Bovine γ-globulin (BGG), lactoferrin (LF), and lactoperoxidase (LPO) were identified as α-Gal carrying proteins which were recognized by AGS patients' IgE. Whey mirrored the anti-α-Gal and IgE reactivity of BGG, LF, and LPO. Eighty-nine percent of the patients displayed IgE to BGG, 91% to LF, and 57% to LPO. Inhibition of α-Gal-specific IgE binding was achieved by BGG, LF, LPO, and whey. These proteins also activated AGS patients' basophils. Interestingly, at lower concentrations, LF was the most potent inhibitor of IgE binding, and the most potent activator of basophils.

Conclusion: BGG, LF, and LPO were all found to be relevant milk α-Gal-containing glycoproteins that bound AGS patients' IgE antibodies and activated their basophils.

Abbreviations: AGS, α-Gal syndrome; AP, Alkaline phosphatase; BGG, Bovine γ-globulin; BSA, Bovine serum albumin; ELISA, Enzyme-linked immunosorbent assay; HSA, Human serum albumin; LF, Lactoferrin; LPO, Lactoperoxidase; ROC, Receiver operating characteristic; RT, Room temperature; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; α-Gal, Galactose-α-1,3-galactose.

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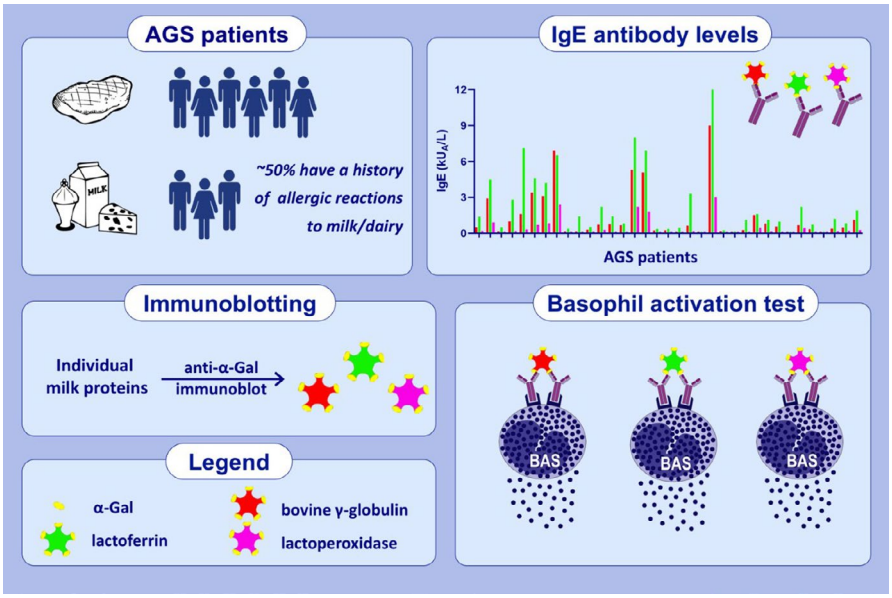
These proteins are probably involved in the allergic reactions to milk in AGS patients. LPO was for the first time shown to be an allergen.

KEYWORDS
bovine γ-globulin, lactoferrin, lactoperoxidase, milk, α-Gal syndrome



GRAPHICAL ABSTRACT

More than half of the AGS patients reported allergic reactions to milk or dairy products. Bovine γ-globulin, lactoferrin, and lactoperoxidase were found to be relevant milk α-Gal-containing glycoproteins that bound AGS patients' IgE antibodies and activated their basophils. These proteins are probably involved in the allergic reactions to milk and dairy products in AGS patients. Abbreviations: α-Gal, galactose-α-1,3-galactose; AGS, α-Gal syndrome; kU$_A$/L, kilo units of allergen-specific IgE per liter.

1 | INTRODUCTION

More than a decade ago, the carbohydrate galactose-α-1,3-galactose (α-Gal) was identified as an IgE epitope that mediates allergic reactions in a novel form of food allergy known as the α-Gal syndrome (AGS).[1,2] The α-Gal epitope is expressed on glycoproteins and glycolipids of non-primate mammals but is also present in some pharmaceuticals. AGS is characterized by late-onset symptoms, usually two to six hours after mammalian meat consumption,[3] which is in contrast to common food allergies where the reactions are immediate. The symptoms range from abdominal pain, urticaria to anaphylaxis, and are often severe. Notably, nearly 50% of the patients experience anaphylaxis.[4] The syndrome typically develops in middle-aged patients who have previously tolerated mammalian meat.[5,6] The induction of the disease has been shown to be associated with tick bites.[7] The first AGS cases were reported in the United States in 2009, shortly followed by reports from Sweden and Australia, and today the syndrome is diagnosed worldwide and its prevalence continues to rise.[1,8,9]

Mammalian meat (beef, pork, and lamb) and especially innards, which are abundant in α-Gal content, are the most common food sources that induce allergic reactions in AGS patients. Other foods that contain α-Gal in lesser amounts are bovine milk and mammalian gelatin.[10–12] Many AGS patients experience allergic reactions upon milk and dairy consumption.[13] IgE reactivity to bovine milk has been observed in more than 90% in our cohort of 128 AGS patients and in nearly 70% in a US cohort of 261 patients.[4,6] Furthermore, a study on 100 randomly selected AGS patients from a US cohort showed that 33% of the allergic manifestations were triggered by dairy.[14] In addition, analysis of a large US cohort of 2500 AGS patients revealed that 10–20% of patients reacted to milk.[15] The most commonly reported symptoms to bovine milk were abdominal pain and urticaria with delayed onset.[16,17]

To date, no milk glycoproteins or glycolipids that carry α-Gal have been identified. This is in contrast to meat, where proteins that carry α-Gal have been thoroughly investigated.[18–20] Kollmann et al have shown that bovine γ-globulin (BGG) is the most prominent carrier of α-Gal and IgE reactive glycoprotein in beef.[19] Interestingly, BGG is present in milk as well.

The aim of this study was to elucidate the importance of bovine milk as an α-Gal-containing food source. The focus was to characterize AGS patients' IgE responses to milk proteins, to identify α-Gal carrying milk glycoproteins, and assess their allergenicity among AGS patients.

2 | MATERIALS AND METHODS

2.1 | Patient cohort and ethics statement

Thirty-eight patients with IgE to milk were selected from our cohort of patients with diagnosed AGS (Table 1). The selection was randomized among almost all available patients (91.4% have IgE to milk[4]). A total of 34/38 answered a questionnaire and were interviewed by the same allergologist with many years of experience in food allergy, regarding milk and dairy consumption and tolerance. Patients' sera were collected and either used individually or as a pool. In addition, three healthy non-atopic and two atopic donors participated in the study as controls. Allergen-specific IgE levels against α-Gal (bovine thyroglobulin), milk, α-lactalbumin, β-lactoglobulin, and caseins were determined by ImmunoCAP (Phadia AB/Thermo Fisher Scientific). IgE antibodies against BGG, LF, LPO, and whey were measured by coupling biotinylated BGG, LF, LPO, and whey proteins to Streptavidin ImmunoCAP (Phadia AB/Thermo Fisher Scientific) according to the manufacturer's instructions. The cutoff for allergen-specific IgE was ≥0.1 kU_A/L. The study was approved by the Swedish ethical review authority (Ethical permit No 2011/1604-31/2, 2018/2483-32) and performed in accordance with the declaration of Helsinki. All AGS patients and controls gave their written informed consent.

2.2 | Materials and reagents

Unprocessed milk was obtained from a local farm in Belgrade. Commercially available pasteurized 3% and 1.5% fat milk were purchased from a Swedish dairy company (Arla Foods). Commercial pasteurized milk was homogenized and pasteurized at low temperature (72–75°C for less than 15 s). Whey was prepared from unprocessed milk as follows. Unprocessed milk was centrifuged at 4000 rpm for 20 min at +4°C, and the upper lipid layer was removed. Caseins were precipitated by lowering the pH to 4.6 by adding 0.1 M HCl and removed by centrifugation at 4000 rpm for 10 min. The resulting whey was further defatted by tetrachlorethylene extraction. Defatted whey was dialyzed against ammonium bicarbonate buffer, lyophilized, and subsequently dissolved in PBS. Protein concentration was determined by the BCA method. The protein concentration of pasteurized milks was 34 mg/ml as per manufacturer's information. Throughout the study, ultra-pure water (18 mΩ) prepared with a Smart2Pure 3 Barnstead aqua purification system (Thermo Fisher Scientific Inc.) was used. Unless otherwise stated, all the other chemicals were purchased from Sigma-Aldrich.

2.3 | SDS-PAGE and immunoblot

Purified milk proteins, α-casein, β-casein, κ-casein, α-lactalbumin, β-lactoglobulin, BGG, lactoferrin (LF), lactoperoxidase (LPO), bovine serum albumin (BSA) (1.5 μg each, Sigma-Aldrich), and whey (15 μg) were separated under reducing conditions on SDS-PAGE electrophoresis at TGX gradient precast gels (any kDa, Bio-Rad Laboratories) using a Mini Protean Cell II system (Bio-Rad Laboratories). Proteins were transferred to PVDF membranes (0.2 μm pore size) using a Bio-Rad Turbo system. Membranes were blocked with 2% human serum albumin (HSA) in phosphate-buffered saline containing 0.05% Tween (t-PBS) for 2 h at room temperature (RT).

The IgE binding to milk proteins and whey was probed by incubating the membrane with a serum pool of AGS patients (#1–13, Table 1; 37 kU_A/L to α-Gal) diluted 1:5 overnight with agitation. For inhibition of IgE binding, the serum pool was preincubated with 150 μg/ml of bovine thyroglobulin for 2 h prior to addition to the membrane. Bound IgE was detected with mouse anti-human IgE labeled with horseradish peroxidase (1:2000, Abcam) for 1 h at RT. Visualization was performed with luminol and H_2O_2 substrates (GE Healthcare) on a ChemiDoc instrument (Bio-Rad Laboratories). Serum from a healthy non-atopic individual (diluted 1:5) was used as control.

To detect α-Gal carrying milk proteins, the membrane was incubated with 1:7500 dilution of chicken anti-α-Gal single-chain antibody variable-region fragment (scFv) (National University of Ireland, Galway)[21] labeled with hemagglutinin tag for 2 h at RT. Subsequently, the membrane was incubated with 0.25 μg/ml mouse monoclonal anti-hemagglutinin antibody (Cat. No. H3663) for 1 h, followed by goat anti-mouse IgG labeled with alkaline phosphatase (AP) (1:1000, Jackson ImmunoResearch Laboratories). AP Conjugate Substrate Kit (Bio-Rad Laboratories) was used for visualization.

2.4 | Inhibition ELISA

The ability of milk and milk proteins (BGG, LF, LPO, and whey) to inhibit IgE binding to α-Gal-HSA was investigated by inhibition ELISA. Microtiter plates were coated with 2.5 μg/ml α-Gal-HSA in coating buffer overnight at +4°C. Individual AGS patients' sera (dilution 1:20) or a serum pool (dilution 1:20) were preincubated with two-fold serial dilutions of α-Gal-HSA (500–3.9 μg/ml), BGG, LF, LPO, whey (1000–7.8 μg/ml), unprocessed milk, and pasteurized 3% and 1.5% fat milk (10 steps of two-fold dilution) for 2h at RT. Thereafter, the remaining IgE binding to plate-bound α-Gal-HSA was detected with rabbit anti-human IgE (1:2000; Miab) for 1 h at RT, followed by AP-labeled goat anti-rabbit IgG (1:1000, Jackson ImmunoResearch Laboratories) for 1 h at RT. Chromogenic 4-nitrophenyl phosphate substrate was used for visualization, and the reaction was stopped with 3 N NaOH. The optical density (O.D.) was read at 405 nm. Inhibition of IgE binding was calculated according to the following equation: % of inhibition = 100 − [O.D. (inhibited) × 100/O.D. (non-inhibited)]. Inhibition ELISA experiments were performed in duplicates and results are represented as average values ±SD.

2.5 | Basophil activation test

Heparinized blood samples were collected from 25 AGS patients (#7–13, 22–39 in Table 1), two atopic, and two non-atopic individuals. Blood aliquots (90 μl) were incubated with stimulants in a

TABLE 1 Data on AGS patients and their specific IgE levels to α-Gal, beef, milk, and milk proteins determined by ImmunoCAP

				Specific IgE ImmunoCAP [kU_A/L]									
Patient no.	Sex/Age	Reactions to mammalian meat	Reaction to milk/dairy	α-Gal	Beef	Milk	α-LA	β-LG	Caseins	BGG	LF	LPO	Whey
1	M/56	GI, U	n.d.	16	9.2	2.4	<0.1	<0.1	<0.1	0.49	1.39	0.18	0.38
2	F/50	AE, U	Tolerant	4.9	0.46	0.11	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
3	F/59	AE, U, GI, ANA	GI	27.3	15.8	5.57	<0.1	<0.1	0.17	2.9	4.48	0.9	1.7
4	M/34	GI	GI	31	1.8	0.31	<0.1	<0.1	<0.1	0.14	0.49	<0.1	<0.1
5	M/53	U	Tolerant	26	24	12	<0.1	<0.1	0.68	9	12	3.0	6.7
6	F/42	AE, U, ANA	GI, U	23	6.8	1.4	<0.1	<0.1	<0.1	0.74	1.4	0.12	0.31
7	F/65	U, GI, ANA	Tolerant	4.9	1.3	0.22	<0.1	<0.1	<0.1	0.16	0.23	<0.1	0.14
8	F/70	U, ANA	n.d.	13	3.8	0.55	<0.1	<0.1	<0.1	0.23	0.35	<0.1	0.29
9	F/42	AE, U, GI, ANA	GI	44	15	1.7	<0.1	<0.1	<0.1	1	2.8	0.18	0.73
10	M/64	AE, U	GI	93	13	2.3	<0.1	<0.1	0.11	1.6	7.1	0.31	1.4
11	M/34	AE, U, GI, ANA	GI	35	13	4.2	<0.1	<0.1	0.19	3.4	4.6	0.7	2.5
12	M/63	AE	Tolerant	40	1.4	0.31	<0.1	<0.1	<0.1	<0.1	0.12	<0.1	<0.1
13	F/29	AE, U, GI	Tolerant	14	4.3	0.46	<0.1	<0.1	<0.1	0.25	1.1	<0.1	0.11
14	F/42	AE, U, ANA	Tolerant	95	25	5.6	<0.1	<0.1	0.35	5.2	n.d.	n.d.	3.1
15	F/34	U, GI	GI	95.2	32.5	5.07	0.1	0.17	0.26	3.1	4.2	0.82	1.4
16	M/52	AE, U, GI, ANA	Tolerant	94	9.7	1.5	<0.1	<0.1	<0.1	0.57	n.d.	n.d.	0.3
17	F/35	U, GI	Tolerant	14	7.6	2.4	<0.1	<0.1	0.14	1.5	1.6	0.43	0.89
18	M/44	AE, U, GI, ANA	Tolerant	34	14	3.8	<0.1	<0.1	0.11	2.2	n.d.	n.d.	1.3
19	M/57	AE, U, GI, ANA	GI, U[a]	100	47	9.2	<0.1	<0.1	0.25	5.1	6.9	1.8	3.4
20	F/69	AE, U	U	31	8.4	1.1	<0.1	<0.1	n.d.	0.45	0.82	0.19	0.28
21	F/37	AE, U, GI, ANA	Tolerant	12	2.8	0.76	<0.1	<0.1	<0.1	0.78	1.1	0.13	0.69
22	F/43	AE, U, GI, ANA	Tolerant	22	6	1.2	<0.1	<0.1	<0.1	0.55	0.98	<0.1	0.57
23	M/37	GI	GI[a]	13	1.2	0.26	<0.1	<0.1	<0.1	0.21	0.36	<0.1	0.12
24	F/60	U, GI	Tolerant	1.2	0.69	0.11	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
25	F/64	AE, U, GI	GI	50	31	10	<0.1	<0.1	0.41	6.9	6.5	2.4	5.7
26	M/62	AE, U, GI, ANA	GI, U	28	12	1.1	<0.1	<0.1	<0.1	0.68	0.79	<0.1	0.46
27	F/45	AE, U, ANA	GI, U	100	26	2.2	<0.1	<0.1	0.43	5.3	8	2.2	4.7
28	M/60	AE, U, GI, ANA	Tolerant	88	34	10	0.21	0.16	0.29	0.68	2.2	0.42	0.61
29	M/67	U, ANA	Tolerant	10	3.9	0.88	<0.1	<0.1	<0.1	0.34	0.72	<0.1	0.25
30	F/29	AE, U, GI	Tolerant	3.2	0.62	0.16	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
31	M/21	U, GI	GI	11	2.6	0.36	<0.1	<0.1	<0.1	0.14	0.37	<0.1	<0.1
32	F/47	U, GI	n.d.	43	4.1	0.37	<0.1	<0.1	<0.1	<0.1	0.44	<0.1	<0.1
33	M/54	U, AE	n.d.	100	25	1.4	<0.1	<0.1	<0.1	0.63	3.3	0.15	0.46
34	F/66	U, AE, GI	GI	21	5.3	0.42	<0.1	<0.1	<0.1	0.13	1.4	<0.1	0.13
35	M/46	GI	GI	14	6.2	0.51	<0.1	<0.1	<0.1	0.3	0.52	0.12	0.26
36	F/56	U, AE, ANA	U	57	23	2.7	<0.1	<0.1	<0.1	1.1	1.9	0.25	0.95
37	M/49	U, GI, ANA	GI	43	13	1.8	<0.1	<0.1	<0.1	0.72	2.2	0.27	0.73
38	M30	GI	Tolerant	17	6.2	0.88	<0.1	<0.1	<0.1	0.37	1.2	0.15	0.35

Abbreviations: α-LA, α-lactalbumin; β-LG, β-lactoglobulin; BGG, bovine γ-globulin; LF, lactoferrin; LPO, lactoperoxidase; ANA, anaphylaxis; AE, angioedema; GI, gastrointestinal symptoms; U, urticarial; n.d., not determined.

[a]Experience symptoms at each exposure to dairy products.

1:1 ratio for 25 min at 37°C. BGG, LF, LPO, whey (0.1–200 μg/ml), and pasteurized 3% fat milk (100–1000 times diluted) were used as stimulants. Anti-FcεRI (Bühlmann Laboratories AG) was used as positive control and PBS as unstimulated control. Cells were stained with FITC-conjugated anti-CD63 and PE-conjugated anti-CD203c monoclonal antibodies (clones CLBGran/12 and 97A6, respectively, Immunotech) at +4°C. Basophils were analyzed by flow cytometry using a BD FACS Canto II (BD Biosciences), and data were analyzed using FlowJo software (Treestar). For all samples, at least 350 basophils were assessed. Basophil activation was calculated as the

percentage of CD63+ out of CD203c+ cells, and 5% CD63+ basophils were set as cutoff for basophil activation.

2.6 | Statistics

Data were analyzed using GraphPad Prism software, version 8. Mann–Whitney *U* test was used for comparisons between the groups. Spearman's rank correlation was used to calculate the strength of an association between two variables. Receiver operating characteristic (ROC) curve analysis was performed to determine the diagnostic value of IgE levels. A *p*-value <0.05 was considered significant.

3 | RESULTS

3.1 | Patients clinical and allergen-specific IgE characteristics

The characteristics of the 38 Swedish AGS patients are shown in Table 1. All patients were IgE positive to α-Gal, beef, and milk. The IgE levels to α-Gal (median 28 kU_A/L; range, 1.2–100 kU_A/L) and beef (median 8 kU_A/L; range, 0.46–47 kU_A/L) were significantly higher ($p < 0.0001$) than those to milk (median 1.3 kU_A/L; range, 0.11–12 kU_A/L) (Figure 1A). Moderate to strong correlations between IgE levels to α-Gal and milk ($\rho = 0.67$, $p < 0.0001$) as well as between beef and milk ($\rho = 0.93$, $p < 0.0001$) were found (Figure S1A,B). Many AGS patients displayed low IgE levels to one or more of the major milk allergens (10 had IgE to caseins and 2 to α-lactalbumin, β-lactoglobulin, and caseins) although none were diagnosed with milk allergy. Based on the questionnaire data, 53% (18/34) of the AGS patients reported a history of allergic reactions to milk and dairy (Table 1). Six percent (2/34) reacted at each exposure to dairy products, while 47% (16/34) experienced reactions at certain exposures only, that depended on the amount consumed, type of dairy products, and cofactors. Reported symptoms were gastrointestinal (89%) and urticaria (33%). AGS patients with a history of allergic reactions to dairy had significantly higher IgE values to α-Gal ($p < 0.05$) compared to patients who were tolerant to dairy products (Figure 1B). There were no significant differences in IgE levels to milk between the two groups (Figure 1B). ROC curve analysis was performed to investigate if differences in α-Gal IgE levels are robust enough to predict the occurrence of allergic reactions in individual patients. The analysis showed that α-Gal IgE levels could indeed discriminate

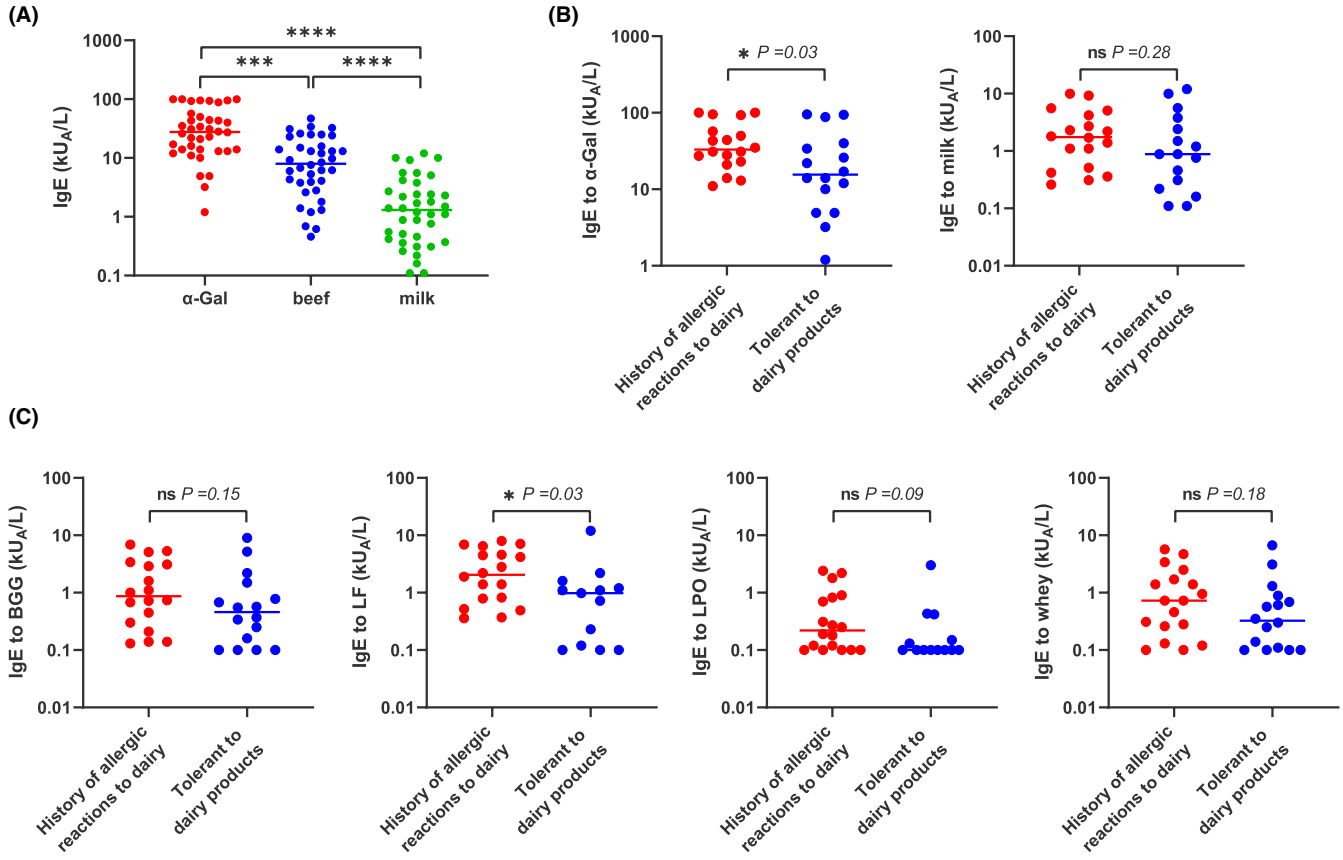


FIGURE 1 Allergen-specific IgE levels among AGS patients. (A) IgE levels to α-Gal, beef, and milk (kU_A/L) ($n = 38$). (B) IgE levels to α-Gal and milk (kU_A/L) among patients with a history of allergic reactions to milk and dairy products ($n = 18$) and patients who are tolerant to milk and dairy products ($n = 16$). (C) IgE levels to BGG, LF, LPO, and whey (kU_A/L) among patients with a history of allergic reactions to milk and dairy products ($n = 18$) and patients who are tolerant to milk and dairy products ($n = 16$). Mann–Whitney *U* test was used for comparisons between the groups. * denotes $p < 0.05$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$, and ns denotes no significant differences

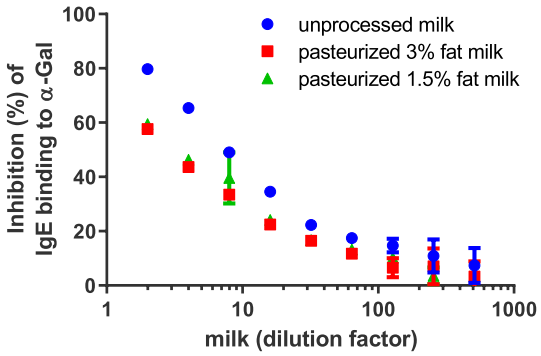


FIGURE 2 Inhibition of patients' IgE binding to α-Gal-HSA by unprocessed and pasteurized milk. The serum pool from AGS patients was preincubated with two-fold dilutions of unprocessed milk, pasteurized 3% and 1.5% fat milk, and the remaining binding to α-Gal was determined in inhibition ELISA. Percentage inhibition of IgE binding is presented

between patients with and without reactions to dairy ($p = 0.03$), but the sensitivity and specificity were not sufficient to make measurement of α-Gal IgE a useful test in the clinical practice for predicting allergic reactions (area under the curve was 0.72).

3.2 | Unprocessed and pasteurized milk inhibit IgE binding to α-Gal

Unprocessed and pasteurized 3% and 1.5% fat milk were assessed for their ability to inhibit AGS patients' IgE binding to α-Gal-HSA. All milk products exerted dose-dependent inhibition of α-Gal-specific IgE binding (Figure 2), showing the presence of α-Gal in unprocessed as well as in pasteurized milk recognized by patients' IgE. Unprocessed milk was able to inhibit IgE binding to α-Gal up to 80%, and pasteurized milks (both 3% and 1.5% milk fat) up to 58%.

3.3 | AGS patients' IgE antibodies recognize BGG, LF, and LPO but not major milk allergens

Next, the most prominent milk proteins α-casein, β-casein, κ-casein, α-lactalbumin, β-lactoglobulin, BGG, LF, LPO, BSA, and whey were selected to be examined for AGS patients' IgE binding in immunoblot using the serum pool. Among the tested proteins, BGG, LF, and LPO were recognized by patients' IgE (Figure 3A). LF was recognized with the strongest intensity. Whey proteins, naturally occurring milk proteins after depletion of caseins, showed multiple bands which corresponded to the molecular weights of BGG, LF, and LPO (Figure 3A). Interestingly, caseins, α-lactalbumin, β-lactoglobulin and BSA, major milk allergens in genuine milk allergy, were not recognized. The serum from a healthy control did not react with milk proteins (data not shown). Preincubation of the serum pool with 150 μg/ml of the α-Gal carrying glycoprotein bovine thyroglobulin, almost completely abrogated the IgE binding to BGG, LF, LPO, and whey (Figure 3B), providing further evidence that the IgE reactivity to these proteins was α-Gal dependent.

3.4 | BGG, LF, and LPO are α-Gal carrying milk proteins

Milk proteins and whey were analyzed for the presence of the α-Gal epitope by performing an immunoblot with the anti-α-Gal antibody (chicken scFv anti-α-Gal). The immunoblot revealed that BGG, LF, and LPO are α-Gal carrying proteins in milk (Figure 3C). Whey proteins showed multiple bands which mirrored the reactivity of purified BGG, LF, and LPO (Figure 3C).

3.5 | IgE antibodies to BGG, LF, LPO, and whey are detected in the majority of AGS patients

IgE reactivity to BGG and whey was assessed in 38 AGS patients and to LF and LPO in 35 AGS patients by ImmunoCAP (Table 1). Eighty-nine percent of the patients were IgE positive to BGG (median 0.6 kU_A/L; range, 0.1–9.0 kU_A/L), 91% to LF (median 1.2 kU_A/L; range, 0.1–12 kU_A/L), 57% to LPO (median 0.13 kU_A/L; range, 0.1–3 kU_A/L), and 82% to whey (median 0.42 kU_A/L; range, 0.1–6.7 kU_A/L). Moderate positive correlations between IgE antibodies to α-Gal and BGG ($\rho = 0.61$, $p < 0.0001$), α-Gal and LF ($\rho = 0.73$, $p < 0.0001$), α-Gal and LPO ($\rho = 0.63$, $p < 0.0001$), and between α-Gal and whey ($\rho = 0.61$, $p < 0.0001$) were observed (Figure S2), providing further evidence that IgE binding to these milk proteins is dependent on α-Gal. AGS patients with a history of allergic reactions to dairy had significantly higher IgE levels to LF ($p < 0.05$) compared to patients who were tolerant to dairy products (Figure 1C). There were no significant differences in IgE levels to BGG, LPO, and whey between the groups, although there was a tendency of higher IgE levels for the group with reported allergic reactions to milk/dairy (Figure 1C). ROC curve analysis showed that LF IgE levels, similarly as to α-Gal IgE levels, could discriminate between AGS patients with and without allergic reactions to dairy ($p = 0.03$), but the sensitivity and specificity were not high (area under the curve was 0.73).

3.6 | BGG, LF, LPO, and whey inhibit AGS patients' IgE binding to α-Gal

The ability of BGG, LF, LPO, and whey to inhibit AGS patients' IgE binding to α-Gal was tested in inhibition ELISA using the serum pool and two individual sera. All tested milk proteins inhibited α-Gal-specific IgE binding in a dose-dependent manner in the serum pool and in individual sera (Figure 4). BGG, LPO, and whey showed similar inhibition pattern, while the pattern of LF differed. LF's inhibition potency was the least dependent on concentration. BGG and whey

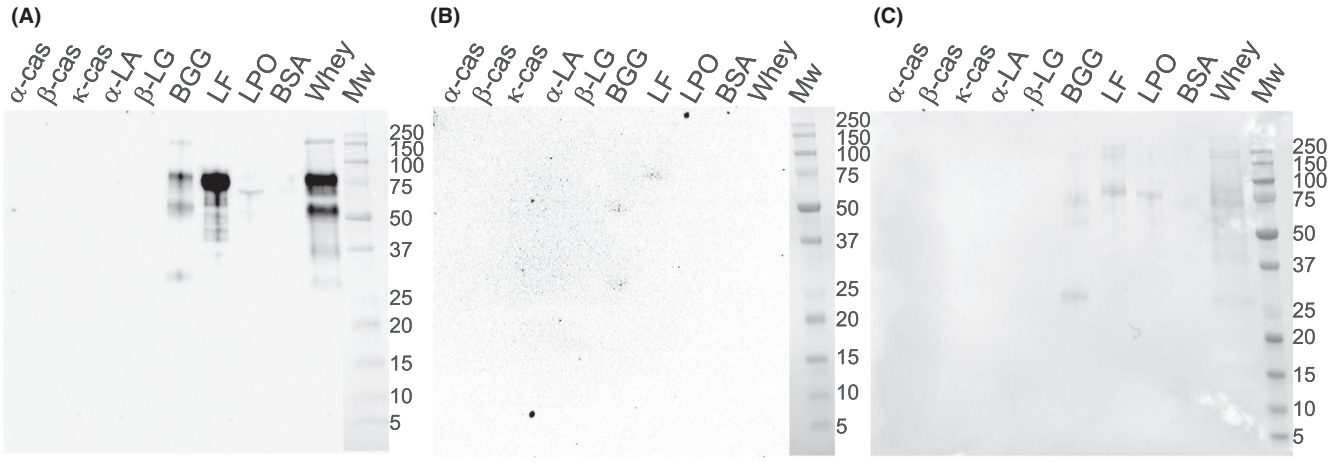


FIGURE 3 Immunoblot analysis of milk proteins. (A) IgE binding to milk proteins and whey in the serum pool from AGS patients. (B) Inhibition of IgE binding to milk proteins and whey by preincubating the serum pool with 150 μg/ml of bovine thyroglobulin. (C) Anti-α-Gal antibody binding to milk proteins and whey. α-cas, α-casein; β-cas, β-casein; κ-cas, κ-casein; α-LA, α-lactalbumin; β-LG, β-lactoglobulin; BGG, bovine γ-globulin; LF, lactoferrin; LPO, lactoperoxidase; BSA, bovine serum albumin; Mw, molecular weight markers (kDa)

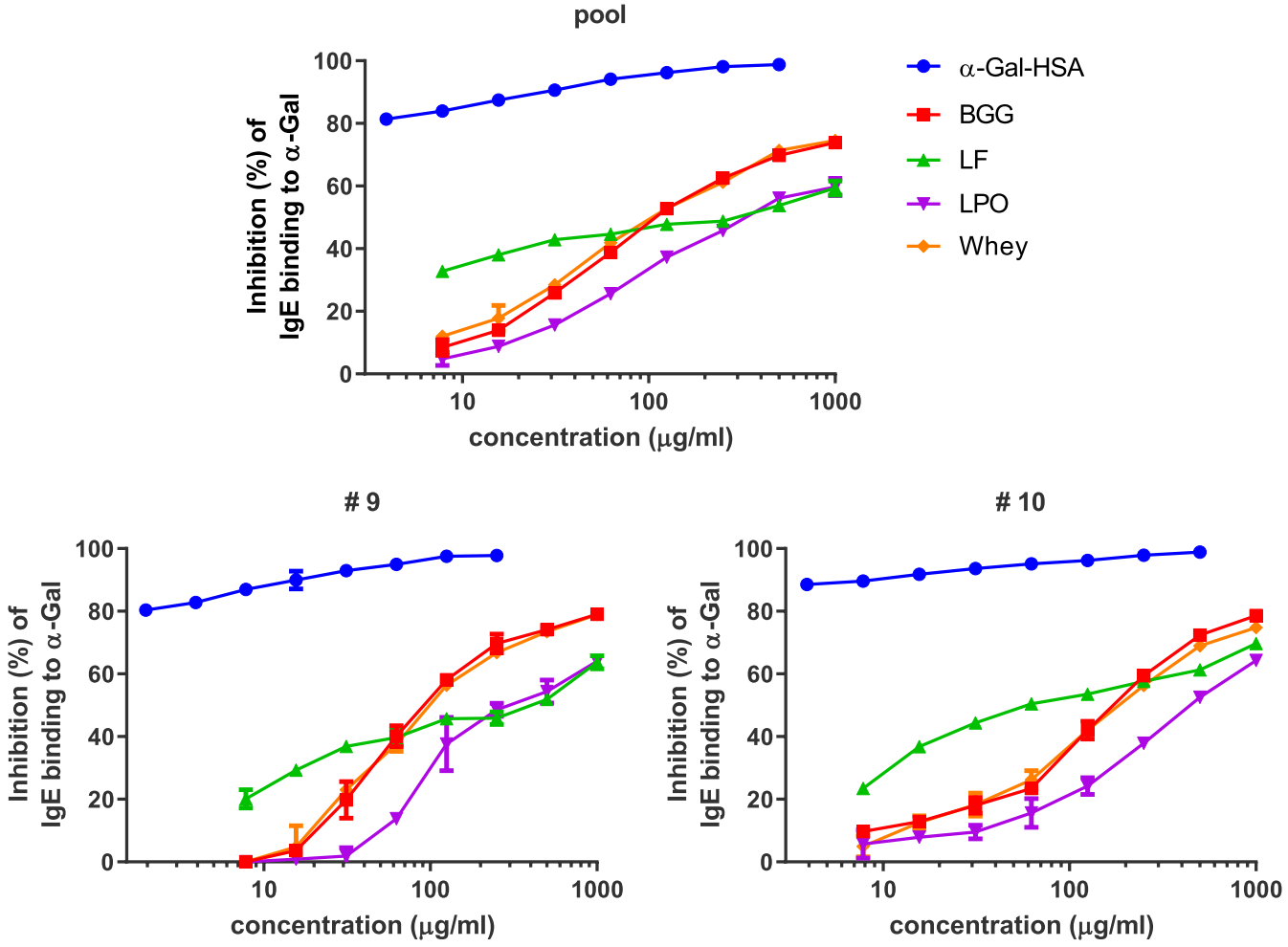


FIGURE 4 Inhibition ELISA. Inhibition of IgE binding to α-Gal by milk proteins and homologous inhibitor. Serum pool or individual sera were preincubated with two-fold dilutions of BGG, LF, LPO, whey, orα-Gal-HSA and α-Gal-specific IgE binding was determined. Percentage inhibition of IgE binding in relation to the concentration of the inhibitors is shown

induced the highest degree of inhibition at the highest concentrations, but LF was the most potent inhibitor of α-Gal-specific IgE binding in the lower range of concentrations. At the highest concentration (1000 μg/ml), BGG achieved inhibition up to 79%, LF up to 70%, LPO up to 64%, and whey up to 79%.

3.7 | Milk, BGG, LF, LPO, and whey activated basophils of AGS patients

The allergenic activity of milk and milk proteins was investigated by basophil activation test among 25 patients with AGS (#7–13, 22–39 in Table 1), two non-atopic, and two atopic controls. Milk and milk proteins activated basophils (>5%) in nearly all patients (milk 23/25; BGG 21/25; LF 7/8, LPO 12/12; and whey 14/18) (Figure 5A,B). LF induced maximum basophil reactivity with lower doses (10 μg/ml) compared to BGG and LPO (100 and 200 μg/ml) (Figure 5B). Interestingly, whey that contains all three allergens showed a reactivity that closely resembled LF. None of the milk proteins activated basophils in control subjects.

4 | DISCUSSION

Bovine milk and dairy products have been reported to induce allergic reactions in AGS patients, and IgE reactivity to milk is common among these patients.[4,13,15] Here, we examined milk and the major milk proteins for their recognition by IgE and allergenicity among AGS patients, as well as for being carriers of α-Gal. We found for the first time that BGG, LF, and LPO are relevant milk allergens for AGS patients. LF was strongly recognized by patients' IgE and was the major α-Gal carrying milk protein. With respect to allergenicity, BGG, LF, and LPO were all shown to activate AGS patients' basophils. We found that more than 50% of our AGS patients had a history of allergic reactions to milk or dairy products, but only 6% reacted at each exposure to milk/dairy. We observed that the IgE levels to α-Gal and LF were significantly higher in the group of AGS patients with allergic reactions to milk/dairy compared to the tolerant group. ROC curve analysis showed that the IgE levels could discriminate between patients with and without reactions to dairy, but the sensitivity and specificity were not sufficient to make measurement of α-Gal or LF IgE levels a useful test in the clinical practice for predicting allergic reactions.

Milk proteins constitute two main protein fractions, caseins and whey. The caseins contain α-casein, β-casein, and κ-casein, and whey proteins contain α-lactalbumin, β-lactoglobulin, BSA, BGG, LF, LPO, and other proteins in minute amounts.[22,23] These milk proteins, except LPO, are known allergens of relevance in genuine milk allergy, of which caseins and β-lactoglobulin are dominating. Our AGS patients with IgE to milk demonstrated different reactivity patterns against the milk proteins. They recognized LF as the major allergen in addition to BGG and LPO. BGG has previously been found as a major IgE reactive protein from beef extract among AGS patients.[19] Many mammalian (eg, murine, equine, ovine, feline) immunoglobulins are known to be carriers of α-Gal, and BGG is one among them.[24–27] To our knowledge, we show for the first time that LPO is an IgE-binding milk protein with allergenic activity. Our findings are in line with the results by Kennedy et al. who showed that caseins, α-lactalbumin, and β-lactoglobulin were not responsible for AGS patients' IgE reactivity to milk.[5]

We thoroughly characterized the AGS patients' IgE binding to purified BGG, LF, and LPO. Nearly all patients displayed IgE reactivity to BGG and LF and more than 50% to LPO. Patients' IgE showed the strongest reactivity with LF (evidenced as the strongest IgE reactive band in immunoblot, and highest IgE levels determined by ImmunoCAP). We noted that the IgE binding to BGG, LF, LPO, and whey was α-Gal-dependent. However, only small amounts of α-Gal are present on their surfaces which are reflected in the lower IgE levels against these proteins compared to α-Gal. The inhibition ELISA experiments also revealed that all three IgE reactive milk proteins

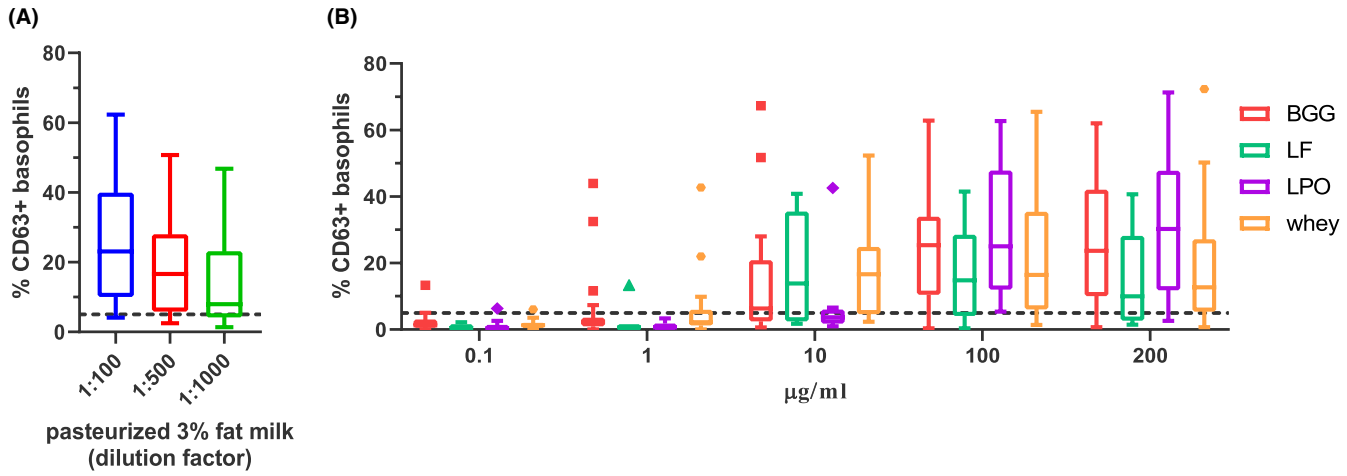


FIGURE 5 Percentage of CD63+ basophils among 25 AGS patients activated by different dilutions of (A) milk and (B) milk proteins. BGG, bovine γ-globulin; LF, lactoferrin; LPO, lactoperoxidase

contain small amounts of α-Gal since high concentrations were needed to achieve inhibition. The inhibition pattern of LF resembled the homologous inhibition of α-Gal-HSA, and among the milk proteins, it was the most potent inhibitor of α-Gal-specific IgE binding at low concentrations. Differences in reactivity patterns of BGG, LF, and LPO suggest that there are several factors influencing α-Gal-specific IgE binding. These can be (a) the amount of α-Gal epitope present on the protein, (b) protein microenvironment in proximity to the α-Gal epitope, (c) representation of α-Gal in the form of mono-, bi-, or tri-antennary glycans,[28] and (d) inhibitory activity of anti-α-Gal IgG, IgA, and IgM antibodies present in patients' serum.[29]

With respect to allergenicity, milk, BGG, LF, LPO, and whey were all shown to induce basophil activation in the majority of AGS patients. The maximum basophil reactivity with LF was achieved at lower doses compared to BGG and LPO. This finding correlates well with LF's higher IgE-binding capacity at lower concentrations. Interestingly, we have previously shown that sensitization to LF is associated with anaphylaxis in AGS patients.[30] Thus, the higher basophil sensitivity to LF might contribute to its role in anaphylaxis. Moreover, the maximum basophil reactivity with milk was achieved at ~10 times higher doses than with meat extract (unpublished data). BGG and LPO induced maximum basophil reactivity at concentrations ~10 times higher than for LF. Purified α-Gal-containing pork allergens, that are considered as the most potent meat allergens, have effective doses approximately 100 times lower.[20] The ability of milk and milk proteins to induce basophil degranulation is in line with the fact that many AGS patients react to milk consumption.

It is important to mention that alpha-Gal carrying glycolipids are assumed to participate in the allergic effector phase in AGS syndrome.[31] They have been demonstrated to bind patients' IgE and activate their basophils.[32] However, alpha-Gal carrying glycolipids from milk remain to be identified and their role in reactions to milk and dairy to be investigated.

In conclusion, BGG, LF, and LPO are major bovine milk α-Gal carrying proteins that are recognized by AGS patients' IgE and activate their basophils. LPO was for the first time shown to be an allergen. Although milk products are not the most potent inducers of allergic symptoms in AGS patients, they contribute to allergic manifestations, especially gastrointestinal symptoms and urticaria.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTION

M.P., D.A., and M.v.H. designed the study. M.P. and D.A. performed the experiments and analyzed and interpreted the data. M.S. collected patients' material and conducted the interviews. M.P., D.A., and M.v.H. wrote the first draft of the manuscript. M.B.G.K., J.G., C.H., and T.C.V. contributed to data interpretation and together with M.S. critically reviewed the manuscript. All authors revised and approved the submitted version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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